SUPPLEMENTAL METHODS AND FIGURES

Abnormal megakaryocyte development and platelet function in *Nbeal2*$^{-/-}$ mice

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Supplemental Methods

Platelet enumeration

Platelet counts were determined for whole EDTA-anticoagulated blood in a Beckman-Coulter ActDiff blood analyzer; mean platelet volumes were determined in diluted samples using a Beckman Coulter LH 750 Hematology Analyzer.

Bone marrow and spleen analysis

Mouse forelegs were dissected and placed in 10% neutral buffered formalin for 24 hours followed by decalcification in 14% EDTA for 72 hours. Whole mouse spleens were placed in 10% neutral buffered formalin for 24 hours. The bone marrow and spleen tissue was then dehydrated through a series of graded ethanol baths and paraffin embedded. Five micron sections were cut and stained with hematoxylin and eosin, and reticulin.

Bright field and electron microscopy

Wright Giemsa-stained mouse blood films were imaged at 100x through a Nikon Microphot SA microscope (Nikon Canada Inc. Instruments, Mississauga, ON, Canada) with a digital camera (Canon 40D). Preparation of platelets for transmission electron microscopy (TEM) was done by centrifuging blood (140g, 15 min) to obtain platelet-rich plasma (PRP), from which platelets were pelleted (800g, 10 min). Following removal of the supernatant, the platelet pellet was fixed with 2.5% glutaraldehyde in PBS pH 7.4 at 4°C for a minimum of one hour or overnight. Bone marrow cells for routine TEM were obtained by flushing mouse femurs with 2.5% glutaraldehyde in PBS pH 7.4 at 4°C and further fixed for a minimum of one hour or overnight. Bone marrow cells prepared for pre-embed labelling were flushed with 0.5% glutaraldehyde in PBS pH 7.4 before carrying out immunolabelling. After this brief pre-fixation step, bone marrow cells for immunoelectron microscopy were washed in 0.1% glycine/PBS followed by 2% BSA/PBS before a one-hour incubation in primary antibody (DakoCytomation rabbit-anti-human VWF, Code No. A0082). The next one-hour incubation in EM goat-anti-rabbit IgG (H&L) 10 nm gold (SPI Supplies, West Chester, PA) was done after repeat rinses in 2% BSA/PBS. Following final rinses in PBS, labeled cells were processed according to routine TEM protocol. All platelets and bone marrow cells including immunolabeled bone marrow cells were post-fixed with 2% osmium tetroxide in dH_2O for one hour and dehydrated in a graded series of acetones before embedding in Epon-Araldite. Thin sections were cut and stained with uranyl acetate and lead citrate. Dense granule evaluation was done on whole mount platelet spreads whereby unfixed platelets within a droplet of PRP were allowed to adhere to the surface of Formvar-coated grids for 3 – 5 minutes. PRP was thoroughly blotted away from the edge of each grid to minimize plasma protein contamination that would have obscured dense granule visualization. Each grid was given a brief one-minute fixation under a drop of 0.5% glutaraldehyde/PBS, dip-rinsed in distilled water and blotted dry. No further processing was necessary before grids were placed in the microscope. Grids were examined with a JEOL JEM-1011 electron microscope at 80 kV; images were captured with a side-mounted Advantage HR CCD camera (Advanced Microscopy Techniques, Danvers, MA) and saved in TIFF format. Digital images were imported into Adobe Photoshop and assembled in Adobe Illustrator for labeling using an Apple Mac Pro computer. Platelet morphometric analyses were done by counting the number of morphologically distinct α-granules in thin sections of 50 WT and 100 *Nbeal2*$^{-/-}$ platelets at a standard

magnification of 40,000x to obtain an average number of α-granules per thin section. Dense granules were counted in 50 WT and 50 *Nbeal2*$^{-/-}$ platelets to obtain an average number per platelet.

Immunoblotting

Preparation of platelet lysates, plasma, immunoblotting and primary antibodies used were as previously described[1] with the exception of the rabbit anti-VPS33B polyclonal antibody (Sigma-Aldrich, Oakville, ON, Canada). For Figure 2A, a 12% reduced SDS-PAGE blot was probed with a mouse anti-TSP1 antibody and subsequently reprobed with a mouse anti-actin antibody. For Figure 2B, a 4-16% reduced SDS-PAGE blot was probed with rabbit anti-PF4 and mouse anti-actin antibodies. For Figure 2C, a 4-16% reduced SDS-PAGE blot was probed for and developed simultaneously using rabbit anti-VWF and mouse GAPDH antibodies. For Figures 2E and 2F equivalent amounts of plasma were run on a 4-16% reduced and a 9% non-reduced SDS-PAGE blot and probed with rabbit anti-VWF and goat anti-fibrinogen respectively. For Figure 2D a 9% non-reduced SDS-PAGE blot was probed with a goat anti-fibrinogen antibody. Figure 2G shows a representative blot of the quantitative P-selectin analysis (see below). For Figure 2H, a 10% reduced SDS-PAGE blot was probed for and developed simultaneously using rabbit anti-VPS33B and mouse anti-GAPDH antibodies. For Figure 2I, a 10% reduced SDS-PAGE blot was probed with mouse anti-VPS16B and mouse anti-GAPDH.

For quantitative P-selectin analysis, platelet lysates were run on a 12% reduced SDS-PAGE and transferred onto nitrocellulose membrane. The membrane was blocked with Odyssey blocking buffer (LI-COR), probed with goat anti-CD62P (Santa Cruz) and mouse monoclonal anti-GADPH (Thermo Scientific) antibodies and the respective anti-goat Alexa 488 and anti-mouse Alexa Fluor 647 (Sigma) antibodies. The fluorescent image was acquired using Typhoon FLA 9500 laser scanner (GE Healthcare). The densitometry analysis was performed using ImageQuant TL 7.0 software (GE Healthcare).

Platelet preparations for immunofluorescence microscopy

Resting platelets were prepared from PRP (above) fixed with 4% paraformaldehyde (15 min); platelets were pelleted by centrifugation (1000g, 8 min), washed with PBS, resuspended in PBS plus 1% BSA and spotted onto poly-L-lysine coated coverslips. Activated platelets were prepared by treating cells prepared for optical aggregometry with thrombin (1 U/mL) for 5 minutes in the presence of abciximab (20 µg/mL, ReoPro, Eli Lilly and Co.) to prevent aggregation, then fixing and spotting onto coverslips. Platelet preparations were incubated with 100% humidity at 37°C for 90 min, rinsed with PBS and immunostained immediately or kept at 4°C.

Megakaryocyte culture

Primary bone marrow cell cultures were established from two femurs and tibias dissected from the same mouse and flushed with PBS. Marrow cells were pelleted (200g, 3 min) and resuspended in DMEM (Wisent Inc.) containing 10% FBS (GIBCO) and 1% Penicillin-Streptomycin (Wisent Inc.) supplemented with 40 ng/mL of rhTPO (gift from Kirin Brewery Company), and incubated (37°C, 5% CO_2) for 3 days before seeding onto coverslips coated with Matrigel (BD Biosciences) diluted 1:6 with DMEM in 12-well plates. MK development was monitored by taking aliquots of cells for fixation and immunostaining on day 5 of culture. MK population expansion was determined by cytospinning native bone marrow and day 5 cultured marrow cells onto microscope slides

prior to fixation and immunostaining for CD41 expression. Cells were imaged at 4x and total numbers were enumerated by counting DAPI-stained nuclei; MKs were scored as CD41-positive cells. Relative ratios of CD41 positive and DAPI positive cells were calculated using Image Pro 6 (Media Cybernetics) software. For MK ploidy analysis, cells from primary bone marrows and day 5 bone marrow cultures were centrifuged at 400g for 10 minutes, incubated with FITC conjugated anti-CD41 antibody (eBiosciences) at 4°C for 30 minutes and then fixed with 0.5% paraformaldehyde at room temperature for 20 minutes. Fixed cells were incubated with propidium iodide solution (50 µg/mL propidium iodide, 200 µg/mL RNase A, 0.1% Triton-X100) overnight and filtered through a 70 µm cell strainer. Flow cytometry was performed using BD FACSCanto (BD Biosciences) and the analysis was performed using FlowJo (Tree Star Inc.).

Immunostaining

Platelets and marrow culture cells were stained with or without permeabilization with Triton X-100 (0.5% for MKs, 0.2% for platelets) using antibodies that are mouse-specific: rat anti-mouse CD41 (eBioscience Inc.), rat anti-mouse LAMP1 (clone 1D4B; Developmental Studies Hybridoma Bank, University of Iowa) or reported to have strong mouse reactivity: goat anti-P-selectin (Santa Cruz Biotechnology Inc.), rabbit anti-von Willebrand factor (Dako), mouse anti-α-tubulin (Sigma-Aldrich), rabbit anti-non muscle myosin IIA (Biomedical Technologies Inc.), rabbit anti-α-tubulin Alexa Fluor 647 conjugate (Cell Signalling Technology). Secondary antibodies were donkey or goat antibodies specific for mouse, rabbit, goat or rat primary antibodies, conjugated with Alexa Fluor 647 (shown as violet in images), 568 or 555 (red), 488 (green) or 405 (light blue; Invitrogen, Life Technologies Corp.). DNA was stained with DAPI (light blue), samples were prepared with fluorescent mounting medium (Dako).

High resolution confocal laser immunofluorescence microscopy

Images were obtained with oil immersion objectives (60x/1.35 and 100x/1.4) using a Quorum spinning disc confocal inverted fluorescence microscope equipped with 4 solid-state lasers (Spectral Applied Research): 405 nm, 491 nm, 561 nm, 642 nm, an Improvision Piezo Focus Drive, 1.5x magnification lens (Spectral Applied Research), a Hamamatsu C9100-13 back-thinned EM-CCD camera and Yokogawa CSU X1 spinning disk confocal scan head with Spectral Aurora Borealis upgrade. Acquisition and image processing (including deconvolution) were done with Perkin Elmer Volocity software (versions 5.5 – 6.1); images were exported to Adobe Photoshop and/or Illustrator for final preparation. Maximum platelet diameter (MPD) was determined by immunostaining platelets for tubulin and myosin IIA and measuring the longest axis of the tubulin cytoskeletal ring using Volocity 6.

In vitro assessments of platelet function

For flow cytometry assessment, washed platelets from heparinized blood were prepared according to a protocol on the Emfret Analytics Web site (www.emfret.com). Subsequently, platelet activation (anti-P-selectin and JONA binding) by ADP (0.12 to 121 µmol/L final concentration, Sigma-Aldrich), or thrombin (2.4 to 12.1 mU/mL final concentration, Roche Diagnostics) was monitored by flow cytometry (BD FACSCalibur). Best-fit sigmoidal dose-response curves (Figure 4 A-C) were generated using Prism assuming a bottom of 0. If the extra sum of squares *F* test of the null hypothesis that the 3 parameters (slope, EC50 and top) are same between the groups was >0.05, then a

shared fit was drawn; if the F test was <0.05, separate lines were drawn. Impedance aggregometry measurement of platelet activation was done as previously reported.[2] Briefly, blood samples from C57BL/6J and *Nbeal2*$^{-/-}$ mice were collected via carotid cannulation into 3.2% sodium citrate or tris-buffered saline (0.1 mL of 20 mmol/L Tris-HCl, pH 7.3, 137 mmol/L NaCl) containing 20 U/mL heparin (Leo Pharma Inc, Thornhill, ON, Canada) (9:1 vol/vol). Aggregation of citrated blood was initiated by 7 µg/mL collagen (Nycomed Pharma) and assessed using a Multiplate analyzer (Diapharma). Light transmission (optical) platelet aggregation was done by pooling blood from 2-3 mice diluted with PBS/ACD plus 3.2% citrate pH 6.1 prior to centrifugation (140g, 10 min). Cells were pelleted from PRP (1000g, 8 min), resuspended in PBS/ACD pH 6.1, pelleted, resuspended in the same buffer, pelleted and resuspended in physiological buffer (Tyrode's pH 7.2 plus 20 mM glucose, 2 mM magnesium chloride and 2 mM calcium chloride) at a concentration of 200 cells/nL. Platelet suspensions were incubated at 37°C (for a maximum of 180 min) prior to transfer of aliquots (190 µL) to glass cuvettes at the same temperature in a Chrono-Log Model 700 optical aggregometer. Samples were stirred at 1000 rpm and an aliquot (10 µL) of thrombin (Recothrom; ZymoGenetics Inc.), CRP or U46619 diluted in PBS was added to start the assay. When performed in this manner the maximum platelet aggregation measured is less than 100% since activated cells form small aggregates rather than the large clumps observed in PRP or blood (where more fibrinogen is available). For measurements of dense granule release, 450uL aliquots of washed platelets at a concentration of 300 platelets/nL in physiological Tyrode's buffer were assayed by lumiaggregometry as per the Chrono-Log manual to obtain peak ATP secretion values relative to an ATP standard (2 nmoles) for stimulation with thrombin (1U) or U46619 (2 µM). Results shown for optical aggregation and lumiaggregometry are for duplicate or triplicate assays.

Tail bleeding assay

Female *Nbeal2*$^{-/-}$ and age-matched WT mice were anesthetized by intraperitoneal injection. Tails were transected 2 mm from the tip and placed into 37°C saline. Blood loss over 40 minutes was determined by collecting 1 mL samples at specified time points as previously described.[3] Briefly, red blood cells from these samples were lysed and the optical density measured at 405 nm was converted into volume of blood using a standard curve.

Intravital analysis of platelet accumulation and activation after laser-induced injury to cremaster muscle arterioles

After laser-injury to the cremaster muscle arterioles, platelet accumulation and activation were measured by high-speed confocal microscopy as previously described.[2,3] Platelet accumulation was expressed as the sum and maximal intensity of X488 (Dylight-tagged anti-GPIbβ, Emfret) which binds platelets. Platelet activation was expressed as the time to half-maximal increase of the ratio of Dylight-647-tagged anti-CD41 Fab fragments, or anti-P-selectin, to Dylight-488-tagged anti-GPIbβ. The increase in Dylight-647-tagged anti-P-selectin in the thrombus over time was also expressed relative to Dylight-tagged anti-GPIbβ accumulation.

Supplemental Figures

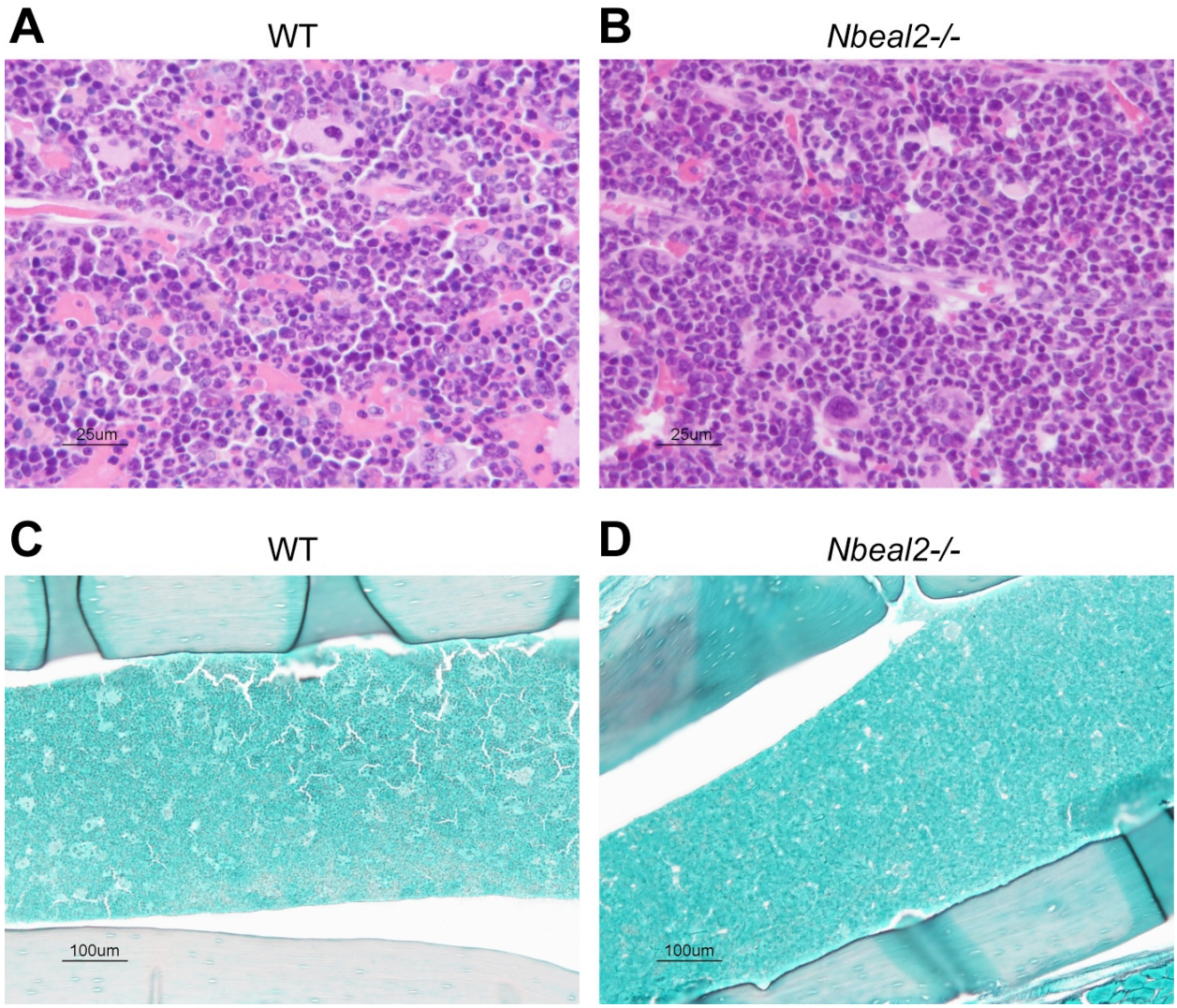
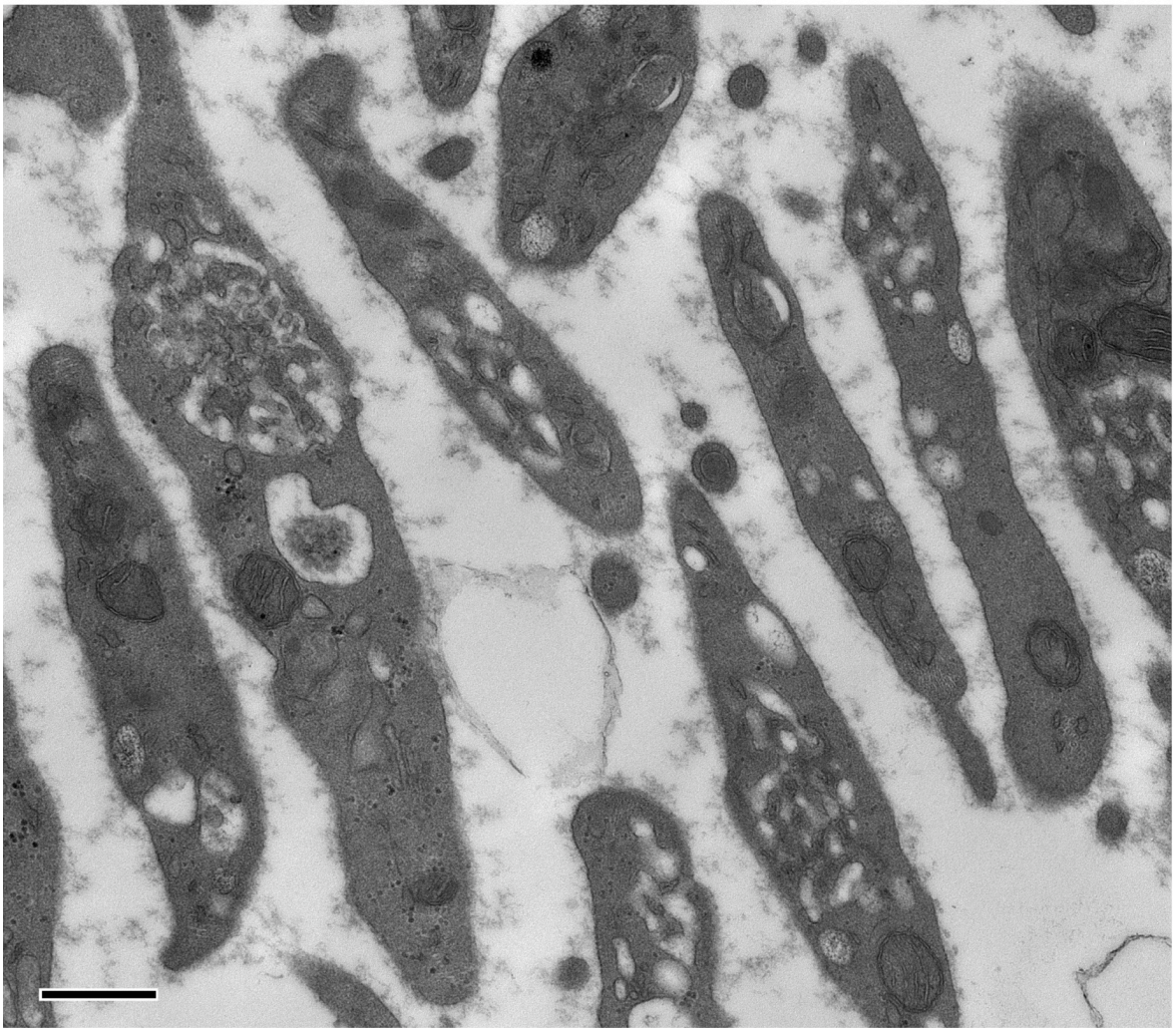
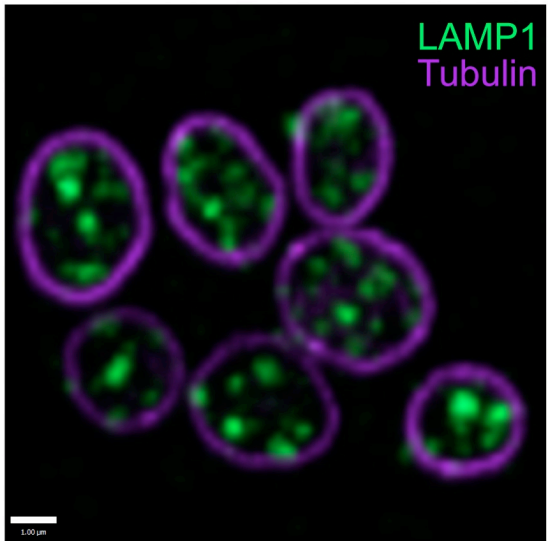


Figure 1. Bone marrow evaluation of *Nbeal2*$^{-/-}$ mice. Bone marrows were prepared from the forelegs of WT (A,C) and *Nbeal2*$^{-/-}$ (B,D) mice and paraffin embedded. Five micron sections were cut and stained with hematoxylin and eosin (A,B), and reticulin (C,D). Both wild type (A) and *Nbeal2*-/- (B) hematoxylin and eosin stained bone marrows revealed normal cellularity and appeared indistinguishable. Similarly there was no abnormality or difference in the reticulin staining in wild type (C) and *Nbeal2*$^{-/-}$ (D) bone marrows, confirming the absence of myelofibrosis in these 4-month old mice (scale bars = 100 μm).

A *Nbeal2-/-*



B WT



C *Nbeal2-/-*

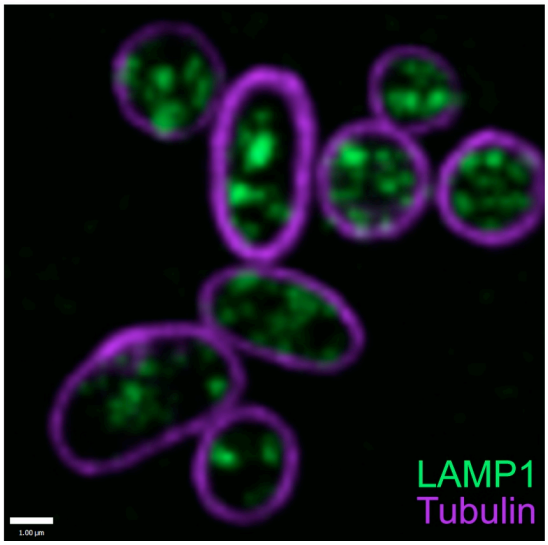
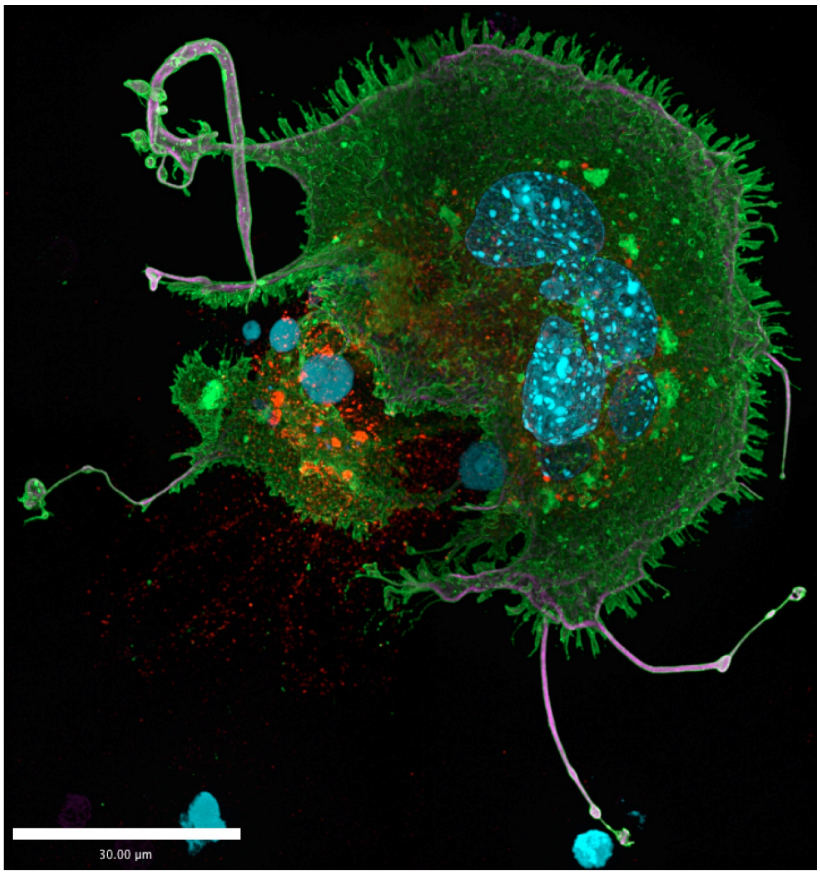


Figure 2. Ultrastructural features and LAMP1 distribution in populations of *Nbeal2*$^{-/-}$ mouse platelets. Thin section transmission electron micrograph of multiple representative *Nbeal2*$^{-/-}$ platelets (A). α-granules were absent in *Nbeal2*$^{-/-}$ platelets (compare to Figure 1D,E). Magnification 30,000x; black bar represents 500 nm. High-resolution confocal laser immunofluorescence microscopy imaging of morphology and intracellular LAMP1 distribution in WT and *Nbeal2*$^{-/-}$ platelets (B,C). Cells were prepared as in Figure 3. The distribution of LAMP1 (green) was similar in WT (C) and *Nbeal2*$^{-/-}$ (D) platelets as shown in the extended focus images of multiple platelets. White bars represent 1 μm.

A *Nbeal2-/-*



B *Nbeal2-/-*

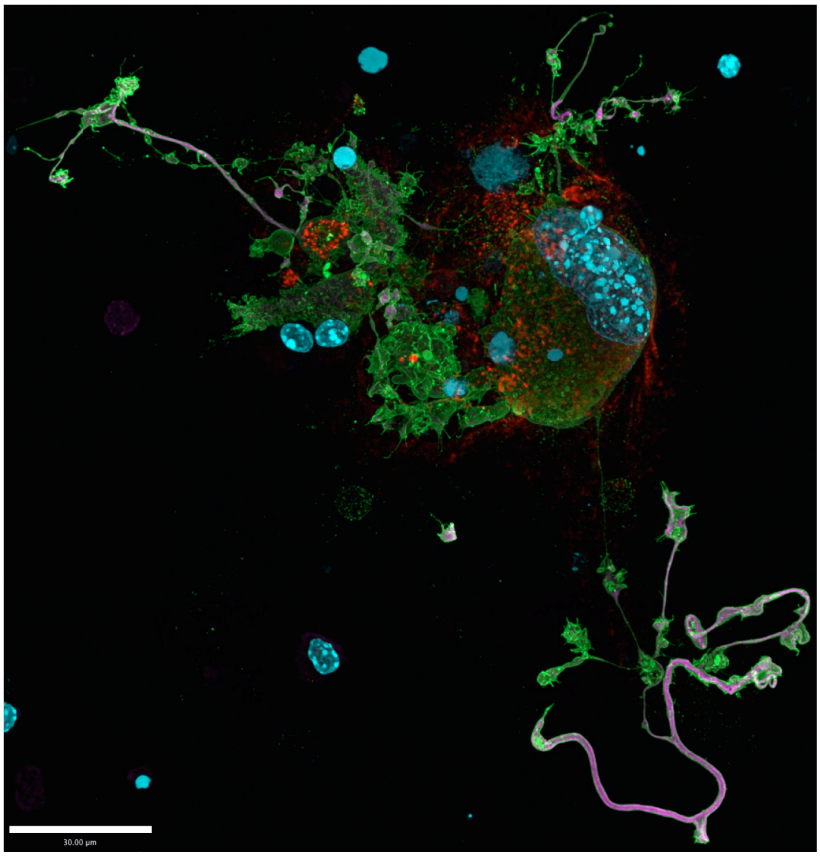


Figure 3. Cultured *Nbeal2*$^{-/-}$ megakaryocyte with proplatelets. MKs were cultured to the terminal proplatelet stage and imaged via immunofluorescence as described in Figure 6. (A) A multilobed nucleus (light blue) is evident in the MK cell with membrane extensions visualized by CD41 (green) and the elaboration of nascent platelets defined by cytoskeletal α-tubulin strands and loops (violet). Extracellular von Willebrand factor (red) is also visible. (B) Large MK producing proplatelets. Colors as in (A). Extended focus image (bar = 30 μm).

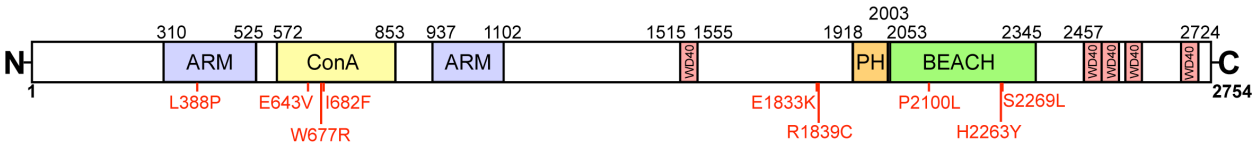


Figure 4. Diagram of NBEAL2, showing functional domains and critical amino acids. Two putative armadillo-like domains (ARM, aa 310-525 & 937-1102), a concanavalin A-like lectin domain (ConA, aa 572-853), 5 WD40 domains (WD40, aa 1515-1555, 2457-2499, 2500-2539, 2551-2592, 2685-2724), a pleckstrin homology domain (PH, aa 1918-2003) and a BEACH domain (BEACH, aa 2053-2345). Several missense mutations causing gray platelet syndrome are illustrated in red.[4-6]

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